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(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: ANDREW, David [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB). LINDSAY, Christopher [GB/GB]; CBDE, Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agents: SKELTON, S., R. et al.; D/IPR, Formalities Section (Procurement Executive), Poplar 2, MOD Abbey Wood #19, Bristol BS34 8JH (GB).

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(57) Abstract

A composition for use in protecting against alkylating agents in particular sulphur mustard; said composition comprising a compound of the formula (I): $H_2NCH(COOR^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1$, or a salt thereof; where R^1 and R^2 are independently selected from hydrogen or an optionally substitued hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, is described. Preferred compounds of the formula (I) are monoisopropylglutathione ester (MIPE) or diisopropylglutathione ester (DIPE). Compositions are suitably formulated as barrier creams and/or liquids for application by nasal spray or inhaler. Method of protecting living tissue from the effects of alkylating agent such as sulphur mustard are also described and claimed.

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Protective Compositions

The present invention relates to the use of certain glutathione ester reagents in the preparation of compositions for protecting personnel against toxic alkylating agents such as sulphur mustard (SM) and carcinogens which bind to thiol groups, to certain compositions obtained thereby, and to methods of protection using these compounds.

The first recorded use of sulphur mustard (HD) as a chemical weapon was during the First World War at Ypres in 1917, during which it was a major cause of military casualties. More recently there have been several instances of its use by Iraq against Iranian and Kurdish targets.

Exposure to HD causes debilitating skin vesication which may result in permanent or temporary incapacitation. Inhalation of HD either in aerosol or vapour form may cause damage or destruction to the respiratory tract, resulting in injury or death. HD is able to form irreversible covalent linkages with many nucleophilic groups in the cell, particularly with DNA. The mono-functional and particularly the bi-functional intra- and inter-strand cross-links formed ultimately result in the inhibition of mitosis and cell death. Exposure to HD also results in the depletion of intracellular NAD^+ levels and consequently glycolysis is inhibited. The enzymes of the pyruvate oxidase system are also sensitive to direct interaction with HD.

Pretreatment of target organs such as lung tissue with nucleophiles may reduce the toxic effects of exposure to HD.

Thiol containing compounds such as cysteine esters have been found to protect rat lung slices *in vitro* (Wilde et al. Human and Experimental Toxicology (1994) 13: 743-748). The use of cysteine compounds as protective compounds is described in WO 92/04024. Glutathione ethyl ester has also been reported to protect peripheral human blood lymphocytes (PBLs) against HD(Gross et al. Proceedings of the 1989 Medical Defence Bioscience Review, US Army Medical Research and Development Command, 15-17 August 1989, Aberdeen Proving Ground, Maryland, USA 415-418 1989),and N-acetyl cysteine can protect human PBLs against the toxic effects of low concentrations of HD *in vitro* (Gross et al., Cell Biology and Toxicology, 1993, 9, 259-267).

In addition, in another context, it has been found (Kobayashi *et al*. Photochemistry and Photobiology, 63, 106-110) that pretreatment of hairless mice with monoisopropylglutathione ester (MIPE) protects against skin damage caused by exposure to UVB radiation.

Such esters have been found to act as suppressors for increased triglycerides in alcohol-damaged livers in rats (JP010063525). Furthermore, pharmaceutical compositions comprising amino acid salts of such compounds have been described in WO 93/25573.

The applicants have found that a different type of glutatione compound produces protective effects which are better than those known hitherto with such compounds.

According to the present invention, there is provided the use of a compound of formula (I)

$H_2NCH(COOR^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1$ (I)

or a salt thereof;
where R^1 and R^2 are independently selected from hydrogen or an optionally substituted hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen; for use in the preparation of a composition for protecting against alkylating agents.

Suitable hydrocarbyl groups for R^1 and R^2 are alkyl, alkenyl, alkynyl or aryl groups such as phenyl or naphthyl, having for example from 3 to 10, preferably from 3 to 6 carbon atoms. Said groups may include straight or branched chains.

Suitable optional substituents for the hydrocarbyl groups include functional groups which do not increase the toxicity and/or solubility of the compound of formula (I) to an unacceptable level, or do not significantly reduce the protective effect of the compounds. Such functional groups may include halogen, such as fluoro, chloro, or iodo, aryl groups such as phenyl, nitro, amino, mono or dialkyl amino groups wherein the alkyl groups suitably contain from 1 to 6 carbon atoms, oxo groups, thio groups, acyl such as acetyl, hydroxy or alkoxy groups such as C_{1-6} alkoxy.

Suitable salts of the compounds of formula (I) are pharmaceutically acceptable salts as is understood in the art.

In particular, the salts may be salts which are soluble in water such as the hydrochloride salt, to assist in formulation.

Preferably, R^1 and R^2 are selected from hydrogen or alkyl, and most preferably C_{3-6} alkyl.

Compounds of formula (I) are glutathione derivatives. Particularly preferred compounds of formula (I) are monoisopropylglutathione ester(MIPE) where R^1 is isopropyl and R^2 is hydrogen, or diisopropylglutathione ester (DIPE) where both R^1 and R^2 and isopropyl groups.

Compounds of formula (I) are known compounds or they can be derived from known compounds by conventional methods, for example by esterification of glutathione.

The compositions produced in accordance with the invention suitably comprise additional reagents as is conventional in the formulation art. These include carriers, adjuvants, fillers, stabilizers, surfactants, emulsifiers etc. The compositions may be in the form of liquids, solids or semi-solids depending upon the intended mode of application.

In particular, the compositions of the invention are in the form of topical compositions for application to the skin. Such compositions include creams and particularly barrier creams. Compositions of this type are novel and form a further aspect of the invention.

Thus the compositions of the invention suitably further comprises a cream base. Examples of cream bases include oil in water emulsions, or polymethoxysiloxane or stearic acid.

Barrier creams for use on the hands may be particularly useful, for example where work has to be done in a contaminated area. Although protective clothing may be effective in maintaining a barrier against HD, it is generally quite cumbersome and may inhibit efficient work. By using a barrier cream, an operator may be able to work without gloves in such an area, at least for a limited period of time.

Alternatively, the compositions obtained in accordance with the invention may be adapted for direct administration to mucosal membranes such as lung tissue and nasal passages. Such compositions may be in the form of nasal sprays or compositions suitable for administration by inhalers or nebulizers such as the Devilbiss nebulizer. In such cases, the compound of formula (I) may be incorporated into structures such as vesicles or liposomes to ensure efficient delivery to the target tissue as are known in the art.

The dosage of compound of formula (I) will depend upon the nature of the patient being treated, the seriousness of the condition or the particular alkylating agent likely to be encountered in accordance with usual clinical practice. In general however, a dosage of from 5 to 60mg/kg, suitably about 30mg/kg would be used. The concentration of the compound within the composition will be appropriate to deliver these dosages in appropriate volumes.

The invention further provides a method of protecting living tissue from the effects of alkylating agent, said method comprising applying to the tissue, a compound of formula (I) as

described above, either before, during or after exposure to said alkylating agent.

Preferably however, the compound of formula (I) or a composition containing it is applied to a subject prior to exposure to the alkylating agent in such a manner that it remains in contact with the tissue during the relevant exposure.

The A549 cell line was used, *inter alia*, as an *in vitro* model to assess the efficacy of potential anti-HD agents, specifically MIPE and DIPE. This line is a human alveolar type II epithelium-like adenocarcinoma derived cell line (Asano et al., Proceedings of the National Academy of Science, USA, 1994; 91, 10089-10093, Wu et al. American Journal of Respiratory Cell and Molecular Biology, 1994: 10, 437-447) and has been used in several studies on pneumocyte biochemistry (e.g Arnold et al. Immunology, 1994, 82, 126-133).

The importance of type II pneumocytes lies in their involvement in alveolar repair processes. In the event of alveolar damage occuring, type II pneumocytes proliferate and replace damaged type I cells; the extracellular matrix secreted by type II cells also influences the repair process (Maniscalco et al., American Journal of Physiology, 1994, 267, L569-L577- Lung Cell Mol. Physiol. 11). The use of nucleophiles to protect type II cells from toxic compounds such as HD may consequently enhance post-exposure healing processes in lung tissue.

Biochemical and morphological approaches were employed to determine both the toxic effects of HD on cells and the protective properties of the above-mentioned esters including MIPE and DIPE against this chemical warfare agent. The

toxicology of HD in A549 cells was determined prior to establishing an appropriate challenge dose for protection studies.

High performance liquid chromatograpy (HPLC) was used to measure the effects of MIPE on the intracellular thiol status of A549 cells.

It was found that HD rapidly reduced the viability of A549 cells at concentrations greater than 40μM, with a 1 h dose of 1000μM HD reducing A549 cell viability to 64% (GV assay) at 10 h. Lower doses of HD increased the time to onset of toxic effects and is an observation consistent with an agent that has numerous intra- and extra-cellular sites of action. The results showed that 200μM HD was an appropriate dose for determining the efficacy of MIPE as a protectant. At this dose of HD, cell growth within the A549 cultures ceased, and cells became detached from the substratum with the remainder being swollen in appearance. Cell death was characteristic of necrosis.

Pretreatment of A549 cells with MIPE (followed by its removal from the cell cultures) resulted in protection, though it produced increased levels of viability ranging between 1.1- and 1.4-fold greater than for HD only exposed cultures. However, higher levels of protection against HD, resulting in increases in viability of up to 2.1-fold compared to HD only exposed cultures, were found when A549 cultures were exposed to HD in the presence of MIPE (co-treatment). These levels of protection are therefore higher than has been reported elsewhere for protective nucleophiles such as GEE (Gross et al. 1989 supra) and NAC (Gross et al. 1993 supra).

The morphology of cells in the protected cultures was normal. Furthermore, MIPE was also effective in protecting A549 cells if added at intervals of up to 7.5 min after the addition of HD. This suggests that HD does not react irreversibly with sensitive biological targets for several minutes, and that within this short window MIPE can effectively "rescue" cell cultures from its lethal effects. CYS levels were found to be raised to high levels by MIPE (comparable to that of non-HD treated cultures) even if MIPE was added 5 min after HD exposure. The protective effects of DIPE were found to be broadly similar to those of MIPE.

HPLC analysis of MIPE treated cultures indicated that the protection which MIPE was found to confer was not due to the raising of intracellular levels of GSH, for these levels did not rise significantly above control levels when cultures were treated with MIPE. In contrast, MIPE was found to raise intracellular CYS levels by up to 40-fold over that of control cultures.

Without being constrained by any particular mode of action, the applicants would speculate that it is more probable that the increased viability of A549 cells pretreated with compounds of formula (I) prior to HD exposure is due to these raised intracellular CYS levels, for other studies have identified compounds that raise intracellular levels of cysteine in rat lungs to levels which protect against perfluoroisobutene-induced pulmonary oedema; these include cystine dimethyl ester, cysteine methyl ester and cysteine isopropyl ester (Lailey *et al*.Biochemical Pharmacology, (1991) 42, S47-S54). Wilde and Upshall (supra.) also found that the methyl, isopropyl, cyclopentyl and cyclohexyl esters of cysteine proved effective

against 0.1 mM HD in protecting rat lung slices *in vitro* against HD. The protection was attributed to the raising of intracellular CYS levels; the interaction between HD and CYS resulting in lower levels of HD being available to exert toxic effects on the tissue.

The increases in viability which were found to occur when co-treating cells with compounds of formula (I) and HD compared to only pretreating with compounds of formula (I) must be due to additional extracellular inactivation of HD by the compound of formula (I), for the intracellular levels of CYS measured in the co-treated cells were comparable to those found in cells pretreated with a compound of formula (I). Increasing intracellular levels of protective nucleophiles such as CYS may therefore not be the only effective strategy against HD.

The inability of MIPE to raise intracellular GSH levels in A549 cells, even though a mechanism exists to transport GSH substrates across the plasma membrane of type II pneumocytes, is surprising. In studies elsewhere, the related compound GEE was found to raise GSH levels in PBLs. GSH protects cells against oxidant injury, and intracellular levels are known to fall in human keratinocytes after exposure to HD. Raising intracellular GSH levels may therefore be an effective strategy against HD toxicity, though the present results suggest that other strategies can be more effective.

The basis of the interaction between HD and compounds of formula (I) is probably due to the presence of the thiol (-RSH) group in the compounds of formula (I). Thiol groups of this type are classified as soft nucleophiles. It is the epi-sulphonium ion (ESI) of the highly reactive cyclic intermediate which is the

active principle of HD, not the β-chloroethyl structure. The ESI intermediate of HD alkylates nucleophilic residues in macromolecules, forming covalent linkages; the nucleophilic substitution reactions commonly proceed via S_N1 and S_N2 mechanisms. Sulphur nucleophiles possess the greatest affinity for the ESI and this may be an important factor in the use of thiol containing compounds to protect cells and tissues from the effects of HD. Compounds of formula (I) appear to be effective scavengers of HD in the biological system described in the present study and this is the first report of its anti-HD protectant properties.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 shows the effect of a 1h pulse of HD on A549 cell viability over time;

Figure 2 illustrates the protection of A549 cells by pre-, co- and post treatment with MIPE;

Figure 3 is a graph illustrating the protection of A549 cells by the addition of MIPE to HD exposed cultures over time;

Figure 4 shows the results of experiments to test the protective effect of compounds of formula (I) on the BEAS-2B and RPMI 2650 cell lines;

Figure 5 shows the results of experiments to test the protection of A549 cells with DIPE where A549 cells were

incubated at 37°C with either HBSS only (I), HBSS + DIPE (II), HBSS + HD (III), and DIPE + HD (IV);

Figure 6 shows the results of experiments to test the protection of A549 cells by cotreatment with DIPE where DIPE (8 mM; 100 µl) was present on the cell cultures at the time of HD challenge (200 µM HD; 100 µl). A549 cells were incubated at 37°C with either HBSS only (I), HBSS + DIPE (II), HBSS + HD (III), and DIPE + HD (IV).

Figure 7 shows the protection of A549 cells by pretreatment and cotreatment with DIPE where HBSS only is (I), HBSS + DIPE (II), HBSS + HD (III) and DIPE + HD is (IV).

Figure 8 shows the protection of A549 cells by post-treatment with DIPE with either HBSS only (I), HBSS + DIPE (II), HBSS + HD (III) and DIPE + HD (IV); and

Figure 9 shows the results of an experiment to test the protection of A549 cells by the addition of DIPE to HD exposed cultures over time.

Example 1

Preparation of Esters

L-Cysteine isopropyl ester hydrochloride

Hydrogen chloride was bubbled through a stirred suspension of L-cysteine (33 0 g, 0.27 mmol) in propan-2-ol (250 ml) and the reaction mixture brought slowly to reflux maintaining the presence of hydrogen chloride. After 5.5 h the reaction was cooled to room temperature and stored overnight. The resultant

solid was filtered off and washed with several quantities of ether to give the pure ester (37.0 g, 68%).

V_{max} (KB,disc)3000(br), 1780(C=O).

^1H δ(CD_3OD): 5.30(1H, sep, J=7.0Hz, OC<u>H</u>), 4.50(1H, m, C<u>H</u>N), 3.30(2H,m, $C\underline{H}_2S$).

^{13}Cδ(CD_3OD): 167.0(C=O), 72.5(O<u>C</u>H), 55.8(<u>C</u>HN), 25.2($\underline{C}H_2S$), 21.8(<u>C</u>H3).

<u>t-Boc-cystine</u>

To a stirred solution of cystine (15.4 g, 64.0mmol) in *tert*-butanol was added NaOH (5.13 g, 128.0mmol) in water (55 ml) followed by di-*tert*-butyl dicarbonate (28 g, 128mmol). After 5 hours, the mixture was extracted with ether (3 x 75 ml), the aqueous phase was adjusted to pH 2 and the resultant solid filtered off and dried to give the pure protected amino acid (15.0 g, 50.5%).

^1Hδ(CD_3OD): 4.40(2H,m,C<u>H</u>N), 3.23(2H,dd,J=4.49 and 10.7Hz,$C\underline{H}_2S$), 2.95 (2H,dd,J=8.70 and 10.7Hz,$C\underline{H}_2S$), 1.44(6H,s, $C\underline{H}_3$).

^{13}Cδ(CD_3OD): 174.1(O<u>C</u>=O), 157.8(N<u>C</u>=O), 80.7(<u>C</u>(CH_3)$_3$), 54.7(<u>C</u>H), 28.7($\underline{C}H_3$).

<u>N-α-t-Boc-L-glutamic acid α-isopropyl-γ-benzyl ester</u>

A solution of N-α-t-Boc-L-glutamic acid-γ-benzyl ester (10.0 g, 29.6 mmol), DBU (4.5 g, 32 mmol), 2-bromopropane (4.0g, 32mmol) and a catalytic amount of tetrabutylammonium bromide (100 mg) in dry THF (150 ml) were heated under reflux. After 9 hours the solvent was removed under reduced pressure and the residue dissolved in CH_2Cl_2 (150 ml). The organic layer was washed with water (100 ml), HCl (1M, 100 ml), $NaHCO_3$ (10% solution, 100 ml), water (100 ml) in turn, dried over anhydrous $MgSO_4$ and concentrated at reduced pressure to give pure product (11.0 g, 98%).

^{1}Hδ($CDCl_3$), 7.30(5H m Ar), 5.1 (2H,s $ArCH_2$), 5.05 (1H,br NH), 5.05 (5H, sept,J = 5Hz,CHO), 4.25 (1H,m(br),CHNH), 2.40 (2H,m(complex),CH_2CH_2C=O), 2.15-1.95 (1H each,m(br) CH_2CH_2C=O), 1.41 (9H,s,$(CH_3)_3$),1.23-1.22 (3H each,d,J=5 Hz $(CH_3)_2$).

N-α-t-Boc-L-glutamic acid α-isopropyl ester

The t-boc protected benzyl ester (7.0 g, 18.5 mmol) was dissolved in THF (150 ml), a catalytic amount of 10% palladium on charcoal added and the mixture hydrogenated until the uptake of hydrogen ceased. The suspension was filtered and the filtrate concentrated at reduced pressure to give pure product.

δ^1H ($CDCl_3$): 5.20 (1H,(br)NH), 5.02 (1H, sept, CHO), 4.20 (1H, br, CHN) 2.50, (2H, m, CH_2C=O), 2.00 (2H, br, CH_2CH_2), 1.41 (9H, s, $(CH_3)_3$), 1.22-1.24 (3H each, 2d's $(CH_3)_2$).

^{13}Cδ ($CDCl_3$): 177.5, 177.1 (C=O, CO_2H and CO_2) 155.5 (NC=O), 80.1 ($OC(CH_3)_3$), 69.4 (CHO), 67.9 (CHN), 30.1 (CH_2CO=O), 25.6 (CH_2CH), 30.1, 28.4 ($(CH_3)_2$), 28.3 ($(CH_3)_2$).

t-Boc-Cystine-Gly-isopropyl ester

To a stirred suspension of t-boc-L-cystine (6.4g, 138 mmol) in dry THF (100 ml) at -10°C N methylmorpholine (3.0 ml, 27.6 mmol) and isobutylchloroformate (3.6 ml, 27.6 mmol) were added. The mixture was stirred at -10°C for 12 min then glycine isopropyl ester hydrochloride (4.3 g, 27.6 mmol) and N-methylmorpholine (3.0 ml, 27.6 mmol) were added and the mixture allowed to warm to room temperature. After stirring for a further 18 h the solvent was removed at reduced pressure and the residue dissolved in a mixture of ethyl acetate (400 ml) and a 10% $NaHCO_3$ (100 ml). The organic layer was washed with water (100 ml), HCl (1 M, 100 ml) and water (100 ml) in turn, dried over anhydrous $MgSO_4$ and concentrated at reduced pressure.

Recrystallisation of the residue from petrol ether / ethyl acetate gave the pure peptide (6.5 g, 69%).

^{1}Hδ ($CDCl_3$): 8.10 (2H,br,CH_2NH), 5.50 (2H,d,J=9.7Hz,CHNH) 5.00 (2H,sep,J=6.2Hz,OCH), 4.90 (2H,t(br),NHCH), 4.10 (2H,dd,J=6.6 and 17.3Hz,NCH_2), 3.80 (2H,dd,J=5.5 and 17.4Hz,NCH_2), 3.07 (2H,dd,J=4.0 and 14.7Hz,SCH_2), 2.90 (2H,dd,J=1.8 and 14.7Hz,SCH_2), 1.40 (18H,s,$C(CH_3)_3$), 1.25 and 1.24 (12H,d,$C(CH_3)_2$).

Cystine-gly-isopropyl ester

Trifluoroacetic acid (20 ml) and triethylsilane (8 ml) were added to a stirred solution of the peptide (6.5g, 10mmol) in dry CH_2Cl_2 (40 ml). After storing at room temperature 15 h the mixture was concentrated at reduced pressure, triturated with ether and the resultant solid washed with several quantities of ether to give the pure unprotected peptide TFA salt (7.0 g, 98%).

^{1}Hδ(CD_3OD): 5.05(2H,sep,J=6.5Hz,$(CH_3)_2CH$), 4.30(2H,m,CHN), 4.0(1H,dd,J=17.5Hz,NCH_2), 3.1,3.45(2H each, SCH_2), 1.25(12H,d,J=6.5Hz,$C(CH_3)_2$).

Boc-glu-cystine-gly-diisopropyl ester

To a stirred solution of t-boc glutamic acid α isopropyl ester (2.6 g, 9.78 mmol) in dry THF (50 ml) at -5°C under argon were added N-methymorpholine (1.1 ml, 9.78 mmol) and isobutylchloroformate (1.3 ml, 9.78 mmol). After 15 min a solution of cystine-gly isopropyl ester TFA salt (2.1 g, 4.8 mmol) in dry THF (10 ml) was added and the suspension brought to room temperature. After stirring for a further 17 h the mixture was concentrated at reduced pressure and the residue dissolved in a mixture of ethyl acetate (300 ml) and 10% $NaHCO_3$ (100 ml). The organic extract was washed with H_2O, HCl (10% solution), and

H_2O in turn then dried over $MgSO_4$ and concentrated at reduced pressure. Purification by column chromatography (silica gel, CH_2Cl_2-MeOH9.8:0.2) gave the pure tripeptide (2.4 g, 51%).

$^1H\delta$(CDC13): 8.44(2H,br,NHCH$_2$C=O), 6.85(2H,d(br)NHC=O), 5.47, (2H,t(br),OC=OCHN), 5.30 (2H,d(br)NHCHCH$_2$S), 5.05 (4H,2septs,J=5Hz,CHO), 4.31(2H,t(br),NHCHCH$_2$S), 4.05(2H,dd,J=18.1, 6.0 HZ(CH$_2$N), 3.88(2H,dd,J=17.9, 4.9Hz,CH$_2$N), 3.05(2H,dd,J=15.5,CH$_2$S), 2.90(2H,dd,J=15,11Hz CH$_2$S), 2.35(4H,t,J=7Hz,CH$_2$C=O), 2.20-1.95(2H each,br,CH$_2$CH$_2$C=O), 1.40(18H,S,(CH$_3$)$_3$), 1.23-1.21(12H each,2d's,J=5.5Hz,(CH$_3$)$_2$).

Glu-cystine-gly-diispropyl ester (oxidised glutathione diisopropyl ester)

Trifluoroacetic acid (5.0 ml) and triethylsilane (2.5 ml) were added to a stirred solution of the protected peptide (1.4 g, 1.43 mmol) in dry THF (10 ml). After 17 h the mixture was concentrated at reduced pressure and the residue triturated several times with pet ether to give the pure deprotected peptide TFA salt (1.4 g, 97%).

$^1H\delta$(CDCl$_3$): 7.38(1H,s(br),NHCH$_2$), 6.92(1H,d,J=8.1Hz,NHCH), 5.01 (2H, two seps,J=6.0Hz,CHO), 4.65 (1H,ddd,J=4.0,5.6,8.3 Hz,CHCH$_2$S), 4.10(1H, dd, J=6.0, 15.9, CH$_2$N), 3.80, (1H,dd, J=4.4,18.1,CH$_2$N), 3.40(1H,dd,J=4.4,9.1,CHNH$_2$), 3.20(1H,dd,J=4.6,13.9,CH$_2$S), 2.67(1H,dd,J=5.7,13.9,CH$_2$S), 2.35(2H,m,J=obs,CH$_2$CHN), 2.10(2H,m,J=obs,CH$_2$C=O), 1.30(12H,d,J=6.0Hz,CH$_3$).

$^{13}C\delta$ (CDCI$_3$): 175.0,172.7,170.1,169.0(4xC=O's), 69.4,68.8(2xCHO's), 54.6(CHNH$_2$); 53.7(CHCH$_2$S), 41.6(CH$_2$N), 33.7(CH$_2$CHN), 29.6(CH$_2$C=O), 26.6(CH$_2$S), 21.8(CH$_3$).

Glutathione diisopropyl ester (reduced glutathione diisopropyl ester)

The oxidised peptide TFA salt (1.4 g, 1.39 mmol) was dissolved in water (10 ml) and the pH adjusted to 7 by the addition of 10%$NaHCO_3$ the solution was stirred at room temperature and dithiothreitol (0.27 g, 1.75 mmol) was added. After 17 h the mixture was analysed by reverse phase HPLC. The column used was a Vydac protein and peptide C18, the mobile phase was solvent A (95% water, 5% TFA) and solvent B (95% acetonitrile, 5% TFA). The solvent programme comprised 0-2 min 5% solvent B, 2-25 min linear gradient of 5-50% solvent B, 25-29 min linear gradient of 40-90% solvent B, 29-32 min 90% solvent B, 32-35 min linear gradient of 90-5% and 35-40 min 5% solvent B at a rate of 1ml/min. The eluate was monitored using a uv dector at wavelengths 224 and 254 nm. The product had a retention time of 17.5 min and there was no evidence of any starting material. The reation mixture was acidified to pH 2 by the addition of 10% HCl, extracted with ether (x3) and the extracts discarded. The aqueous phase was adjusted to pH 7.5 and extracted with ether (x8). The combined extracts were dried over $MgSO_4$ and concentrated at reduced pressure to give the title compound (0.66 g, 48%).

Glutathione diisopropyl ester

To a stirred solution of glutathione (25.0 g, 81.4 mmol) in sodium dried propan-2-ol (250 ml) under argon at room temperature concentrated H_2SO_4 (12.5 ml) was added dropwise. After storing at room temperature 3 days the mixture was analysed by HPLC, conditions as above. The chromatogramme showed the presence of 4 products identified as both monoesters, the diester and a cyclised product. The mixture was

concentrated at reduced pressure, the pH adjusted to 7.5 by the addition of 10% $NaHCO_3$ and extracted with ether (x3). The extracts were combined, dried and concentrated at reduced pressure to give the ester slightly contaminated with oxidised material. The impure ester was dissolved in a minimum amount of water, the pH adjusted to 7 by the addition of 10% HCl and excess dithiothreitol added. After 17 analysis by HPLC, conditions as above, showed the reaction to be complete. The mixture 7 was acidified to pH 2 extracted with ether (x3) and the extracts discarded. The aqueous phase was then adjusted to pH 7-8, extracted with ether (x3) and the combined extracts dried over $MgSO_4$. Concentration at reduced pressure gave the title compound (5.1g, 15.8%).

Glutathione mono-isopropyl H_2SO_4 ester

To a stirred solution of glutathione (10.0 g, 32.6 mmol) in propan-2-ol(sodium dried, 100 ml) under argon at room temperature, concentrated H_2SO_4 (2.74 ml) was added dropwise. After stirring at room temperature 18 hrs the mixture was diluted with diethyl ether (sodium-benzophenone dried, 100 ml) stored at -4°C to crystallise glutathione mono-isopropyl H_2SO_4 ester. After 2 days the mixture was filtered and the crystals washed with diethyl ether (3 x 100 ml) to give the title compound (14 g, 93%).

$^1H\delta(CD_3OD)$: 5.05(1H,sep,J=6.0Hz,CHO), 4.56(1H,dd,J=5.5,7.0Hz,CHCH$_2$S), 4.07(1H,m,CHNH$_2$), 3.90(2H,dd,J=17.7Hz,CH$_2$NH), 2.87(1H,dd,J=5.5,14.0Hz,CH$_2$S), 2.84(1H,dd,J=7.3,14.0Hz,CH$_2$S), 2.60(2H,t,J=7.4Hz,CH$_2$C=O), 2.20(2H,m,CH$_2$CH$_2$C=O), 1.25(6H,d,J=6.0Hz,CH$_3$).

$^{13}C\delta(CD_3OD)$ 174.5, 172.8, 171.4, 170.6(4x<u>C</u>=O'S), 70.3(<u>C</u>HO), 57.1(<u>C</u>HCH_2S), 53.6(<u>C</u>HNH_2), 42.4(<u>C</u>H_2N), 32.3(<u>C</u>H_2CHN), 27.0(<u>C</u>H_2C=O), 25.2(<u>C</u>H_2S), 22.0(<u>C</u>H_3).

Example 2

Cell Cultures

The A549 cell line (no. 86012804) was obtained from the European Collection of Animal Cell Cultures, Centre for Applied Microbiology and Research, Porton Down, Salisbury, UK.

The cell line growth medium consisted of DMEM containing FCS (10%, v/v), penicillin G (100 IU/ml), streptomycin (100 mg/ml), and glutamine (2 mM); this is referred to subsequently as DMEM medium. Cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C. They were harvested using trypsin/EDTA in log growth phase for use in toxicology studies.

The A549 cells were seeded into 96-well plates at a density of 5 x 10^4 cells/well in DMEM medium. The cells were allowed to adhere over a period of 24 hours (h) prior to treatment with HD. Exposure to HD and to MIPE was carried out in the 96-well plates. HD dilutions in HBSS were prepared immediately beforehand from a stock solution of 40 mM HD in propan-2-ol.

Example 3

<u>Determination of the effect of pulses of HD on A549 cells</u>

The onset of HD-induced toxicity in A549 cell cultures was determined by measuring the viability of cultures over time using the genetian violet (GV) assay. Cellular density in A549 cultures was determined using GV (Gilles et al., Analytical Biochemistry, 1986; 159: 109-113) and was used as a cell viability assay as described by Griffiths *et al* Toxicology,

1994: 90: 11-27; the contents of which are incorporated herein by reference.

To determine the effects of exposure to HD, the DMEM medium overlying the cells was removed and solutions of HBSS (100µl) containing 0 to 1000µM HD were added to each well. The cultures were exposed to the HD solutions for 1 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. The HD solutions were removed and 100µl of DMEM medium added. The cultures were further incubated for periods of up to 72 h under the conditions described above. At the end of each time point, the GV dye assay was carried out to determine the viability of the cultures. Viability under experimental conditions was related to that of control cells exposed to HBSS only (the reference 100% viable cell control). The data is presented in Figure 1 as mean values +/- SD (n=10). In this Figure, results using HBSS solutions containing 8(▲), 40(●), 200(▼), 400(◆) and 1000(■)µM HD are illustrated.

The results show that at 10 h, the interpolated viability of cultures exposed to 1000, 400, 200, 40 and 8 mM HD was 64%, 76%, 77%, 93% and 91% respectively; at 30 h it decreased further to 24%, 37%, 43%, 71% and 84% and at 50 h had fallen to 14%, 24%, 27%, 74% and 80%. The onset of toxic effects was therefore a function of HD dose, with a rapid onset at high concentrations of HD and a slower onset at low concentrations of applied HD.

Example 4

The protection of A549 cells by MIPE

The ability of MIPE to protect A549 cells exposed to an LC_{50} dose of HD was determined by removing DMEM medium from cell cultures, followed by adding MIPE in HBSS to the wells of each 96 well plate. HBSS +/- HD was added to the wells after, during and before treatment with HBSS +/- MIPE. A total of 48 wells/plate were used. The cultures were incubated with these solutions for 1 h each at 37°C (5% CO_2/95% air), after which the solutions were replaced with 100µl of DMEM medium and further incubated at 37°C for a total of 24 and 48 h, at which times viability was determined by both GV assay described above, and also the NR cell viability assay.

The NR assay was used in the present study as an index of viability and is described in detail by Griffiths et al. Toxicology, 1994, 90: 11-27, the content of which is incorporated herein by reference. M199 medium was used when employing the neutral red (NR) dye system to avoid possible interference from the phenol red present in DMEM. The dye accumulates in the lysosomes of viable cells.

Four sets of experiments were conducted in which the treatments were added in the following combinations: (a) the MIPE was put on the cultures for 1 h then removed and the cell layer washed once with 100µl HBSS, prior to the addition of HD for a further 1 h; (b) the MIPE was present on the cell cultures at the time of HD challenge; (c) the HD was added to the cultures, followed by the immediate addition of MIPE for a further 1 h; (d) the

cultures were incubated with HD for 1 h, followed by the addition of MIPE for a further 1 h. The effects of these treatments was compared to cultures treated with HBSS, MIPE and HD alone (12 wells per treatment). The final concentration per well was 8 mM for MIPE and 100μM for HD (total volume; 200μl). NR and GV stained cultures were photographed using an inverted microscope equipped with phase contrast optics at a magnification of x200.

In order to probe the efficacy of immediate post-treatment of HD-exposed cells with MIPE, a further set of experiments was carried out to determine if adding MIPE at various times after the application of HD to the cultures would reduce the toxicity of HD. In this work, 100 μl HD (200μM) was added to the wells of a 96-well plate, and at intervals of up to 15 min, 100 μl MIPE (16 mM) in HBSS was then added to give final concentrations of 100 μM HD and 8 mM MIPE. The effect of this treatment was compared to cultures treated with HBSS, MIPE and HD alone. The cultures were treated with these solutions for a total of 1 h at 37°C (5% CO_2/95% air), after which the solutions were removed. The cell layer was washed with 100 μl HBSS and 100 μl of DMEM medium added to the wells and further incubated as above. Viability was subsequently determined at 48 h by the GV assay.

For both these experiments illustrative data are presented in Figure 2 as mean values +/- SD (n=12). A Student's two-tailed t-test (unpaired) was used to determine if any increase in protection obtained by treating cultures with MIPE before during or after HD exposure was significant compared to HD only treated cultures.

Using the NR assay, it was found that cultures of A549 cells treated with MIPE (final concentration 8 mM) during exposure to HD (final concentration 100μM) were 1.8- and 1.9-fold more viable than HD treated cultures at 24 h and 48 h respectively (Figure 2a(1)). This was similar to the results obtained with the GV assay, where the viability of the cultures treated with MIPE prior to exposure to HD was 1.6- and 2.1-fold greater at 24 h and 48 h respectively compared to cultures treated with HD only (Figure 2b(1)). All these increases in viability were found to be extremely significant ($p<0.0001$).

When cultures were treated with MIPE for 1 h which was then washed off, with HD being subsequently added, lower levels of protection were found with cultures being 1.3-fold ($p<0.001$) and 1.4-fold ($p<0.0001$) more viable (NR assay) at 24 h and 48 h respectively (Figure 2a(2) than HD only treated ones. These lower levels of protection are reflected in the results for the GV assays in which the viability of cultures was 1.1-fold (difference not significant) and 1.2-fold ($p<0.05$) greater at 24 h and 48 h respectively (Figure 2b(2)).

A549 cultures were also treated with MIPE immediately after addition of HD. High levels of viability were measured using the NR assay with culture viability being 1.4- and 2.4-fold greater at 24 h and 48 h respectively compared to HD-only treated cultures (Figure 2a(3)). This was similar to the results obtained with the GV assay, where the viability of the cultures treated with MIPE after prior exposure to HD was 1.5- and 2.0-fold greater at 24 h and 48 h respectively compared to cultures treated with HD only (Figure 2b(3)). All these increases in viability were found to be extremely significant ($p<0.0001$).

Addition of MIPE after 1 h HD exposure of the cells to HD resulted in small increases in viability of 1.3-fold using the NR and GV assays at 24 h ($p<0.005$). No increase in viability was measured with either assay at 48 h compared to HD only treated cultures (results not shown).

Photomicrography in particular of the GV stained cultures confirmed the results of the biochemical analyses. Cell cultures treated with HD and no MIPE were substantially depleted of cells, and contained many cells of abnormal, swollen appearance which did not appear to be dividing. The cells in A549 cultures protected with MIPE at the time of exposure to HD exposure, whilst being of lower density than control cultures had a normal morphology, and were comprised of actively dividing cells. The control cultures treated with HBSS + MIPE (8μM) had the same morphology as control cultures treated with HBSS only.

Example 5

Determination of the intracellular non-protein thiol status of A549 cells by HPLC following treatment by MIPE and HD

The effect of MIPE treatment upon the intracellular reduced glutathione (GSH) and cysteine (CYS) status of A549 cultures grown in 75 cm^2 culture flasks was determined by HPLC according to a method described by Lailey *et al*.Biochemical Pharmacology, (1991) 42, S47-S54. In these studies, the DMEM medium from A549 cell cultures was removed and the cell layers washed by washing them with 3 x 10 ml HBSS prior to any treatments being carried out.

The biochemical basis of protection of A549 cultures by MIPE against HD was measured by treating cultures of A549 cells for 1 h at 37°C (5% CO_2/95% air) with: (i) 10 ml HBSS; (ii) 10 ml MIPE (8 mM); (iii) 5 ml MIPE (16 mM) immediately followed by the addition of 5 ml HD (200µM); (iv) 10 ml HD (100 µM). The effect of delayed application of MIPE following HD exposure on the intracellular thiol status of the cultures was determined by treating cultures with (v) 5 ml HD (200µM) for 5 min, followed by addition of 5 ml MIPE (16 mM); these cultures were incubated for a total of 1 h as above. Prior to harvesting, the cell layers were washed as described above. The cell layers were scraped off the substratum in the presence of 900µl of a buffer (50 mM N-ethylmorpholine/HCl; pH 8.0) containing 0.5 mg/ml monobromobimane. The cell suspension was homogenised and incubated in the dark for 2 h at 20°C. 100µl of an aqueous solution of sulphosalycylic acid (6%, w/v) was added to the suspension and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatants were decanted and filtered through 0.2µm filters into HPLC vials. The method was based on the derivatization of thiols with monobromobimane to form fluorescent groups. The supernatant samples which contained the adducts were injected via a Perkin Elmer (PE) ISS200 autosampler onto a 0.46 cm x 15 cm, 3µm ODS C_{18} column equipped with a Corasil guard column. The ODS column was coupled to a PE LC250 binary high pressure liquid chromatography (HPLC) pump working at 3000 psi. Elution was stepped using 91% of a mixture A (0.25% glacial acetic acid, 99.75% HPLC water; adjusted to pH 3.7 with NaOH (0.1 M)) and 9% acetonitrile for 7 min followed by 60% of mixture A, and 40% acetonitrile for 10 min. A 10 min re-equilibration with the initial eluent was allowed before injection of subsequent samples. Fluorescent derivatives were detected on a PE LS-4 spectrofluorimeter coupled to an Amstrad PC3386 operating PE

Nelson software package 2100. The GSH and CYS concentrations of the samples were measured against standard curves of up to 100µg for each thiol compound. The concentrations of GSH and CYS in the samples from both studies were normalised against total protein (determined by the Pierce Micro BCA Protein Assay Reagent Kit, product no. 23235). For each treatment, the results are presented as mean values +/- SD (n=5). Statistical significance was determined by Welch's approximate two-tailed t-test.

The results are shown in Table 1.

Table 1

	CYS		GSH	
	(µg/mg protein)			
Treatment:				
i) HBSS	0.447 +/- 0.092	-	32.6 +/- 2.4	-
ii) MIPE	16.0 +/- 1.9	p<0.0001	34.1 +/- 2.9	NS
iii) MIPE + HD	17.9 +/- 1.2	p<0.0001	36.7 +/- 6.7	NS
iv) HD	0.298 +/- 0.055	p<0.05	27.5 +/- 3.5	p<0.05
v) HD + MIPE	17.9 +/- 1.8	p<0.0001	33.4 +/- 2.0	NS

For each treatment, the results are presented as mean values +/- SD (n=5); NS = not significant.

As can be seen from these results, pretreatment of A549 cells for 1 h with MIPE was found to raise intracellular CYS levels by 35.8-fold (p<0.0001) (Table 1). In cultures treated with MIPE in combination with HD, intracellular levels of CYS remained high (40-fold greater than in control cultures; p<0.0001). Treatment of A549 cultures with HD reduced CYS levels by a factor of 0.67-

fold ($p<0.05$). Intracellular CYS levels were raised by MIPE (36.8-fold greater than control levels; $p<0.0001$) after adding it to cells 5 min after they had been exposed to HD.

MIPE pretreatment of A549 cells for 1 h did not significantly raise intracellular concentrations of GSH. Similarly, MIPE pretreatment of cells prior to HD exposure and co-treatment of cultures simultaneously with MIPE and HD for 1 h did not significantly increase intracellular glutathione levels compared to control cultures. Exposure to HD only decreased intracellular GSH levels by 0.84-fold ($p<0.05$) compared to control cultures.

Example 6

Maintainance of the viability of A549 cell cultures by MIPE after periods of exposure to HD

In order to probe how effective MIPE was at maintaining the viability of cultures exposed to HD, A549 cultures were treated with 100µl HD (200µM). The same volume of MIPE (16mM) in HBSS (IV) or with HBSS only (III) was subsequently added to the cultures at intervals of up to 15 min. Control cultures were treated with 200 µl HBSS only (I) or 200µL MIPE (8mM) in HBSS(II). After incubation for 1h at 37°C (5% CO_2/95% air), the solutions were removed and the cultures were further incubated in DMEM medium and viability determined at 48h using the GV assay. The results for up to the 12.5 min interval are shown in Figure 3. (Data points represent mean values, +/- standard deviation, n=12).

The viability of the cultures exposed to HD for 0.5, 2.5 and 5 min prior to treament with MIPE was 2.1-, 1.9- and 2.0-fold greater respectively than the HD only treated cell cultures ($p<0.0001$). After 5 min, cell viability declined, so that at 7.5

min it was 1.5-fold greater ($p<0.0001$) than that of unprotected cultures. After 7.5 min, cell viability in MIPE treated cultures fell to levels comparable to that of HD only exposed cultures.

Example 7

Protection of Alternative Cell lines

Using similar methodology to that described in Example 4 above, the protective effects of MIPE and DIPE (di-isopropyl ester of glutathione) was tested on two different cell lines. These were the BEAS-2B, a human bronchial epithelial cell line (virally transformed) and RPMI 2650, a human nasal epithelial cell line (probably derived from a tumour).

Cells were cultured as described in Example 2 above and cell viability was measured using neutral red (NR) and gentian violet (GV) assays.

Cells were exposed (in 96-well plates) to a solution of protectant compound (MIPE or DIPE) for 1 hour, a solution containing sulphur mustard was then added. Protectant and sulphur mustard were removed after a further hour, cells were cultured for 48 hours before viability assays were performed. By way of comparison, cells were treated either with mustard alone, or with protectant compound alone. Control cells were exposed to HBSS.

The results are shown in Figure 4 hereinafter. In these tables, the first column represents the viability of cells treated with HD alone, the second column represents cells treated with both HD and protectant, the third column represents cells treated with protectant alone and the fourth column is the control group.

In Figure 4A, BEAS-2B cells were pretreated with MIPE (100 µg/ml) for 1 hour, then exposed to 50 µM sulphur mustard for 1 hour. Viability was measured after 48 hours by gentian violet assay.

In Figure 4B, RPMI 2650 cells were pretreated with DIPE (100 µg/ml) for 1 hour, then exposed to 100 µM sulphur mustard for 1 hour. Viability was measured after 48 hours by neutral red assay.

In Figure 4C, BEAS-2B cells were pretreated with DIPE (100 µg/ml) for 1 hour, then exposed to 30 µM sulphur mustard for 1 hour. Viability was measured after 48 hours by gentian violet assay.

These results clearly show that the viability of all cells treated with either protectant is improved as compared with untreated cells.

Example 8

The Protection of A549 cells by DIPE

The methodology of Example 4 was repeated using DIPE in place of MIPE. In this case, five sets of experiments were therefore conducted in which the treatments were added in the following combinations: (a) the DIPE (4 mM; 200 ml) was put on the cultures for 1 h then removed and the cell layer washed once with 100 ml HBSS, prior to the addition of HD (100 mM; 200 ml) for a further 1 h; (b) the DIPE (8 mM; 100 ml) was present on the cell cultures at the time of HD challenge (200 mM HD; 100 ml); (c) DIPE (8 mM; 100 ml) was put on the cultures for 1 h after which HD (200 mM; 100 ml) was added for another 1 h (in

the presence of DIPE); (d) HD (200 mM; 100 ml) was added to the cultures, followed by the immediate addition of DIPE (8 mM; 100 ml) for a further 1 h; (e) the cultures were incubated with HD (100 mM; 200 ml) for 1 h, followed by the addition of DIPE (4 mM; 200 ml) for a further 1 h. The effects of these treatments was compared to cultures treated with HBSS, DIPE and HD alone (12 wells per treatment). The final concentration per well was 4 mM for DIPE and 100 mM for HD (total volume; 200 ml). GV stained cultures were photographed using an inverted microscope equipped with phase contrast optics (magnification; x200).

Using the NR and GV viability assays as described above, and the thiazolyl blue (MTT) assay as described by Griffiths et al., Toxicology, 1994, 90: 11-27, it was found that cultures of A549 cells pretreated with DIPE for 1 h (and subsequently removed) prior to treatment with HD were 1.2-, 1.4- and 1.3-fold more viable respectively than HD treated cultures ($p<0.0001$) at 48 h (Figure 5).

When cultures treated with DIPE were immediately exposed to HD, the viability of the cultures was 1.6-, 1.5- and 2.0-fold greater at 48 h as determined by the MTT, NR and GV assays respectively, compared to cultures treated with HD only ($p<0.0001$; Figure 6).

A549 cultures were also treated with DIPE for 1 h prior to the addition of HD for a further 1 h (without the DIPE being removed). High levels of viability were measured using the MTT, NR and GV assays; protected cultures were 2.6-, 2.9- and 2.3-fold more viable respectively at 48 h than HD only treated ones $p<0.0001$; Figure 7).

High levels of viability in A549 cell cultures were also measured when they were treated with DIPE immediately after the addition of HD to the cultures. The viability of these cultures as determined by MTT, NR and GV assays was 1.6-, 1.6- and 2.1-fold greater respectively than HD only treated cultures ($p<0.0001$; Figure 8). The addition of DIPE 1 h after exposure of the cells to HD resulted in no increase in viability being measured by the MTT assay, and increases of only 1.1- and 1.3-fold with the NR and GV assays respectively, compared to HD only treated cultures (results not shown).

Photomicrography of GV stained cultures confirmed the results of the biochemical analyses. A control culture of A549 cells at 48 h was photomicrographed as was cell cultures treated with HD and no DIPE. This culture was substantially depleted of cells, and contained many cells of abnormal, swollen appearance which did not appear to be dividing. The cells in A549 cultures protected with DIPE at the time of exposure to HD, whilst being of lower density than control cultures had a normal morphology, and were comprised of actively dividing cells. The control cultures treated with HBSS + DIPE (8 mM) had the same morphology as control cultures treated with HBSS only.

Example 9

Maintainance of the viability of A549 cell cultures by DIPE after periods of exposure to HD

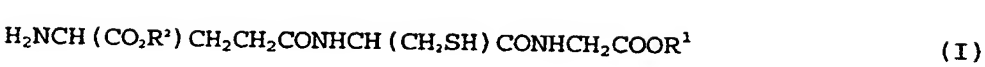
In order to probe the efficacy of immediate post-treatment of HD-exposed cells with DIPE, a further set of experiments was carried out to determine if adding DIPE at various times after the application of HD to the cultures would reduce the toxicity of HD. In this work, 100 ml HD (200 mM) was added to the wells of a 96-well plate, and at intervals of up to 20 min, 100 ml

DIPE (8 mM) in HBSS was then added to give final concentrations of 100 mM HD and 4 mM DIPE. The effect of this treatment was compared to cultures treated with HBSS, DIPE and HD alone. The cultures were treated with these solutions for a total of 1 h at 37°C (5% CO_2/95% air), after which the solutions were removed. The cell layer was washed with 100 ml HBSS and 100 ml of DMEM medium added to the wells and further incubated as above. Viability was subsequently determined at 48 h by the NR assay. For both these experiments the data are presented as mean values +/- SD (n=12). Welch's approximate two-tailed t-test (unpaired) was used to determine if any increase in protection obtained by treating cultures with DIPE before during or after HD exposure was significant compared to HD only treated cultures.

The results are shown in Figure 9. The viability of the cultures exposed to HD for 0.5, 2.5 and 5 min prior to treatment with DIPE was 1.8-, 1.5- and 1.4-fold greater respectively than the HD only treated cell cultures ($p<0.0001$). After 5 min, cell viability declined, so that at 7.5 and 10 min it was 1.4- and 1.3-fold greater ($p<0.0001$) than that of unprotected cultures. After 12.5 min, cell viability in DIPE treated cultures fell to levels comparable to that of HD only exposed cultures.

Claims

1. The use of a compound of formula (I)



$$H_2NCH(CO_2R^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1 \quad (I)$$

or a salt thereof;

where R^1 and R^2 are independently selected from hydrogen or an optionally substituted hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, in the preparation of a composition for use in protecting against alkylating agents.

2. The use according to claim 1 wherein R^1 and R^2 are selected from hydrogen or alkyl.

3. The use according to claim 2 wherein said alkyl group is a C_{3-6} alkyl.

4. The use according to any one of claims 1 to 3 wherein the compound of formula (I) is monoisopropylglutathione ester (MIPE).

5. The use according to any one of claims 1 to 3 wherein the compound of formula (I) is diisopropylglutathione ester (DIPE).

6. The use according to any one of the preceding claims wherein the composition is in a form which is suitable for topical application.

7. The use according to claim 6 wherein the composition is in the form of a barrier cream.

8. The use according to any one of claims 1 to 5 wherein the composition is in liquid form.

9. The use according to claim 8 which is in a form suitable for administration by a nasal spray or by an inhaler.

10. The use according to claim 9 wherein the compound of formula (I) is incorporated into vesicles or liposomes.

11. A method of protecting living tissue from the effects of alkylating agent, said method comprising applying to the tissue, a compound of formula (I) as described above, either before, during or after exposure to said alkylating agent.

12. A method according to claim 11 wherein said compound is applied before or during exposure to said alkylating agent.

13. A method according to claim 12 wherein said compound is applied before and during exposure to said alkylating agent.

14. A pharmaceutical composition comprising a compound of formula (I)

$$H_2NCH(CO_2R^2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COOR^1 \quad (I)$$

or a salt thereof;
where R^1 and R^2 are independently selected from hydrogen or an optionally substituted hydrocarbyl group having at least three carbon atoms; provided that R^1 and R^2 are not both hydrogen, and

a pharmaceutically acceptable carrier, wherein the composition is in a form suitable for topical administration.

15. A composition according to claim 14 which comprises a barrier cream.

Fig.1.

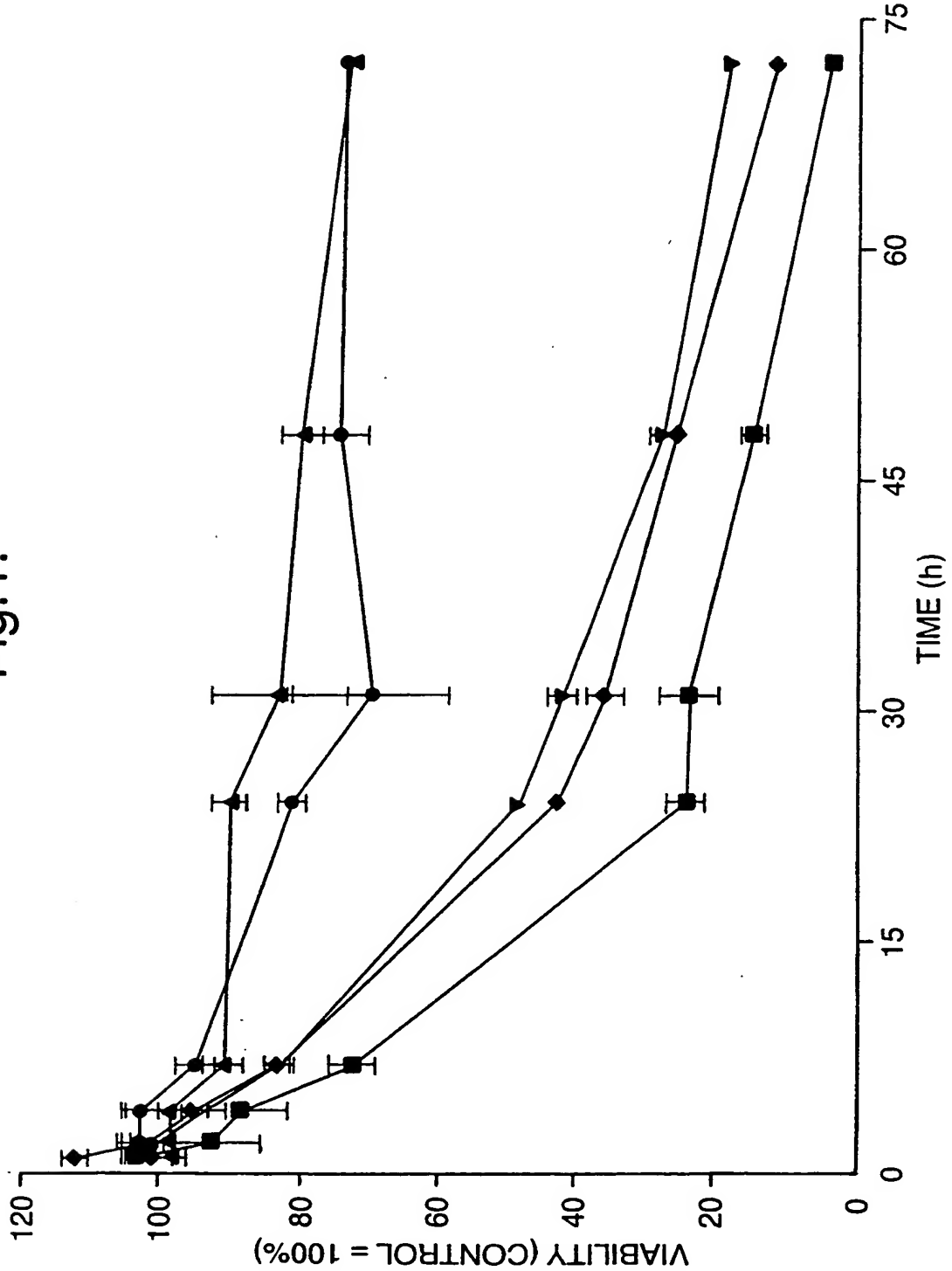


Fig.2a.

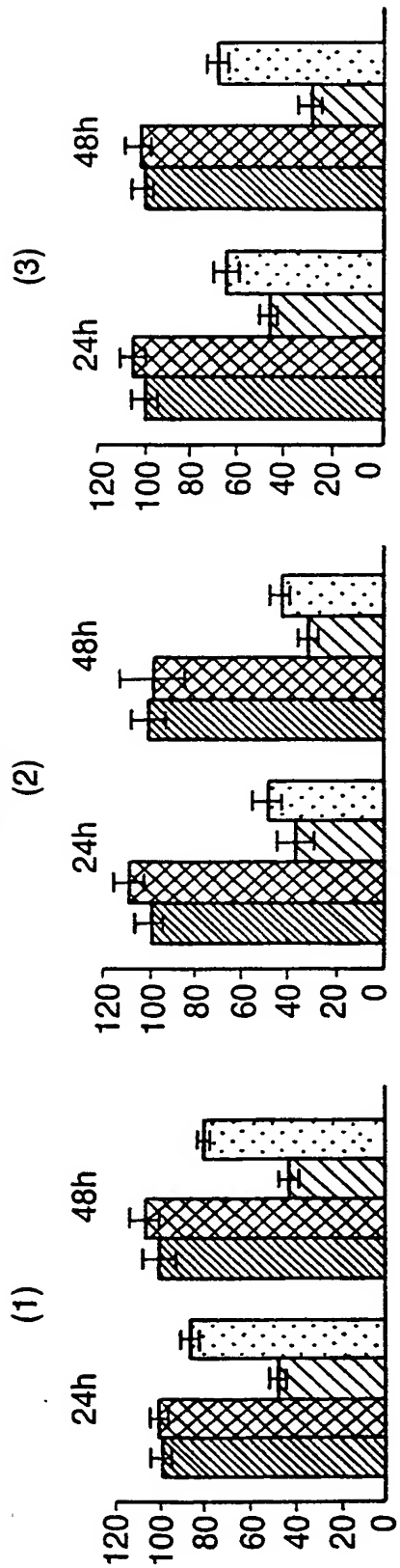


Fig.2b.

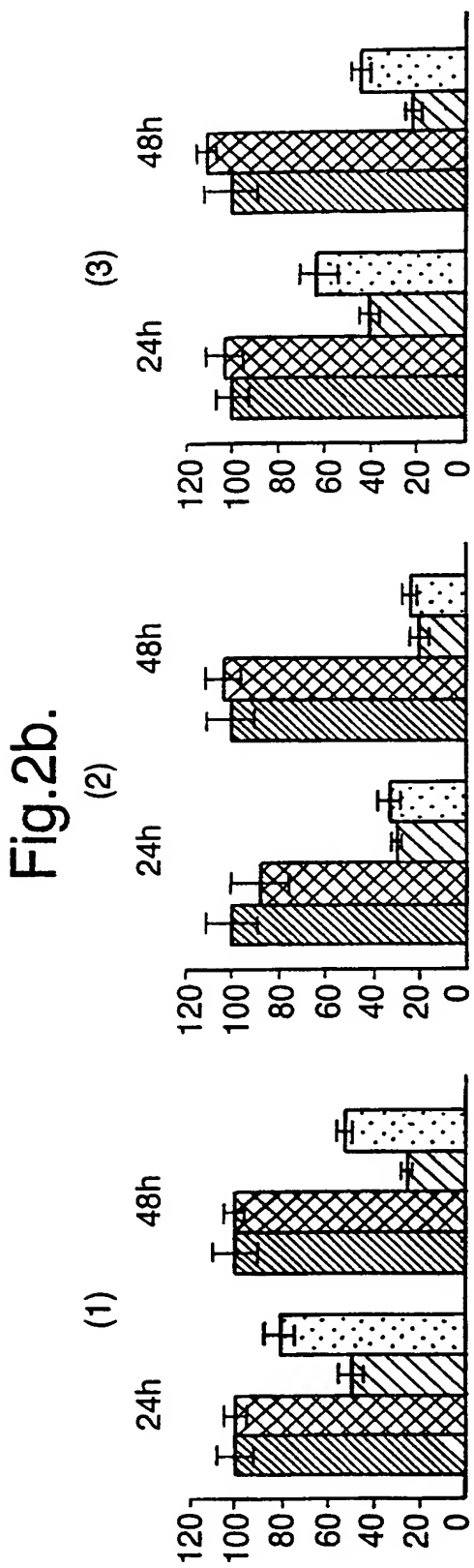


Fig.3.

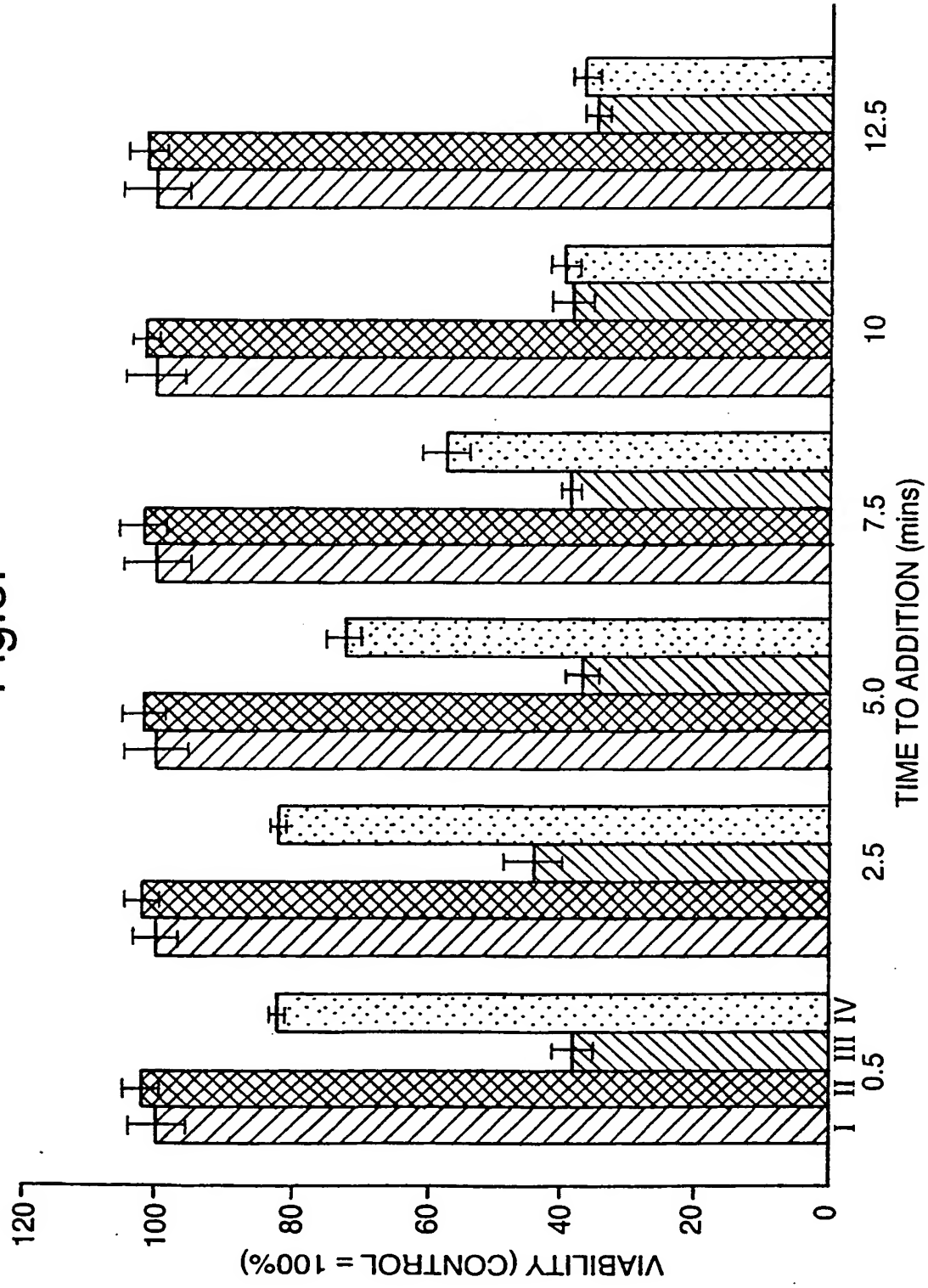


Fig.4A.

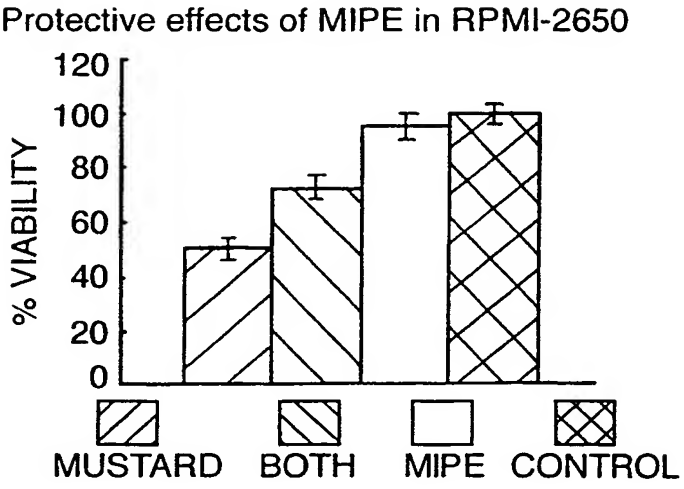


Fig.4B.

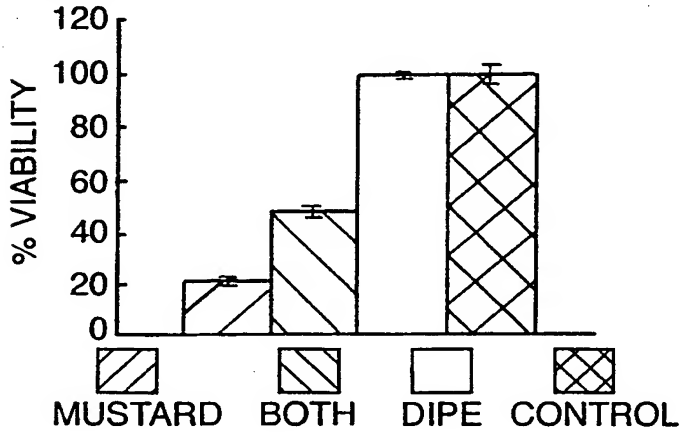


Fig.4C.

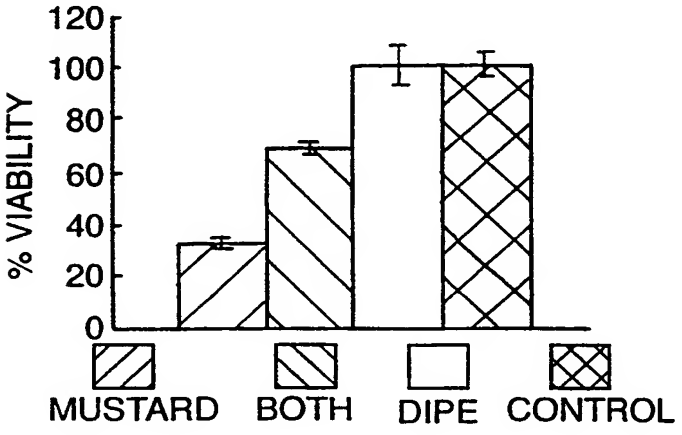


Fig.5.

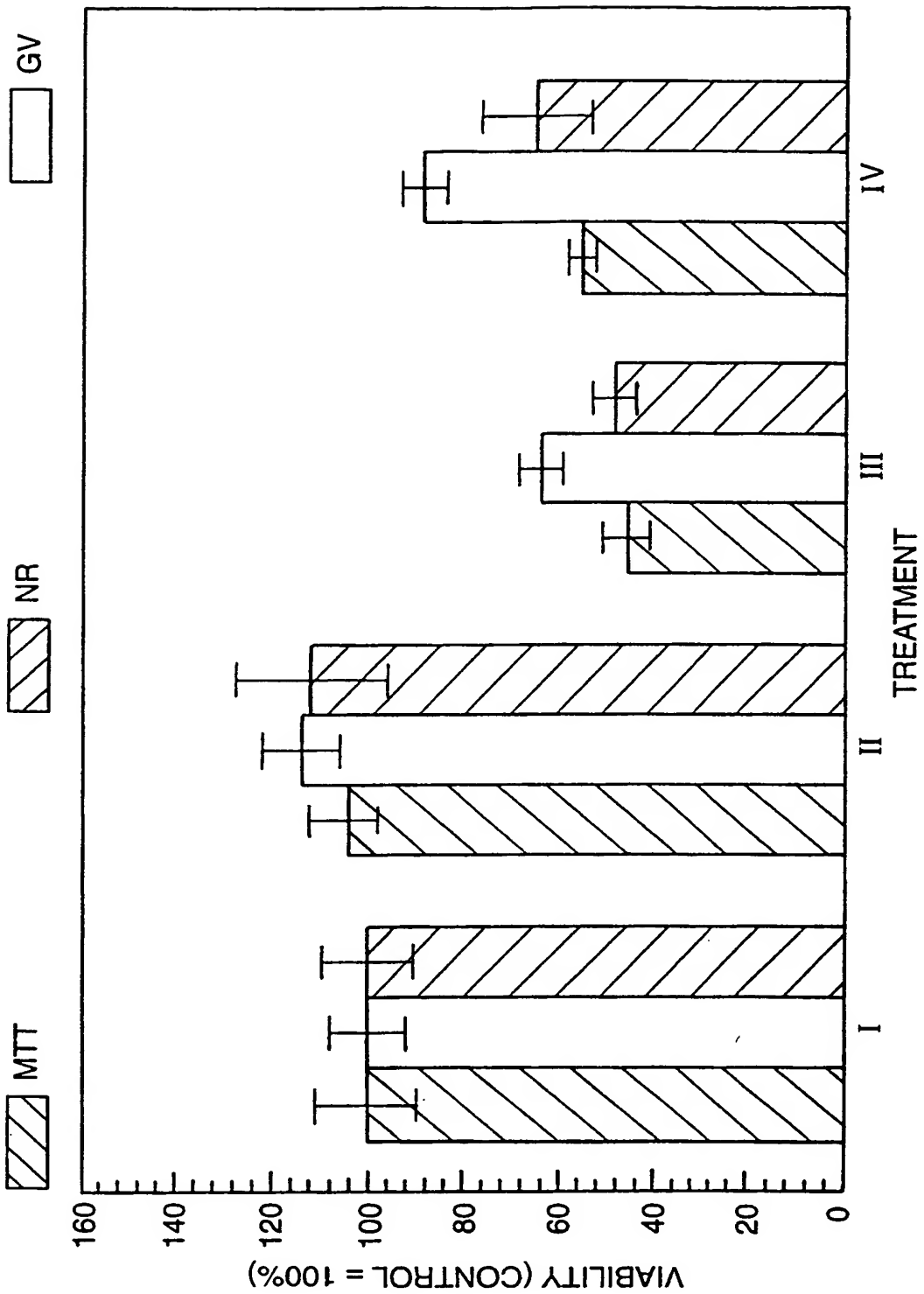


Fig.6.

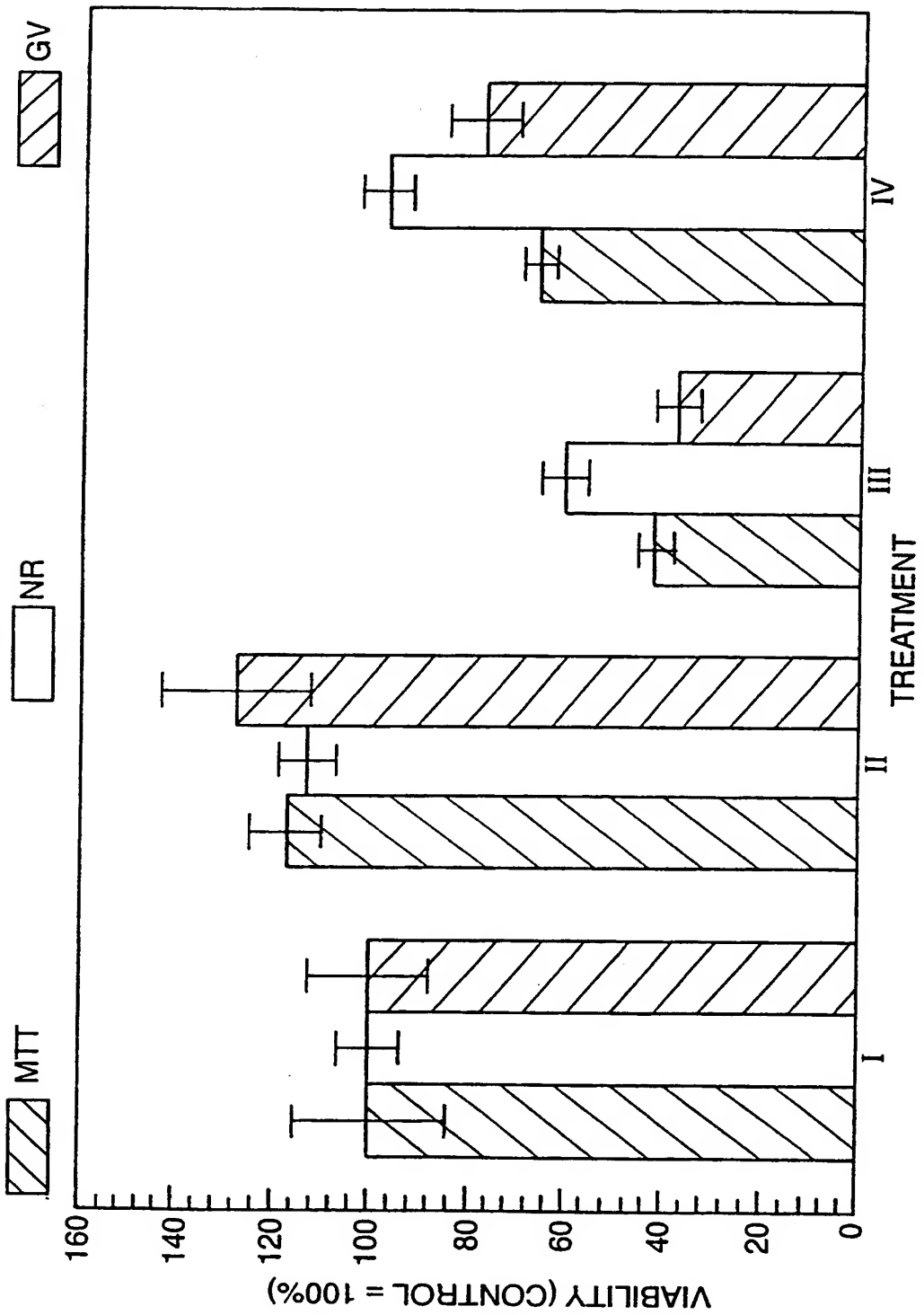


Fig.7.

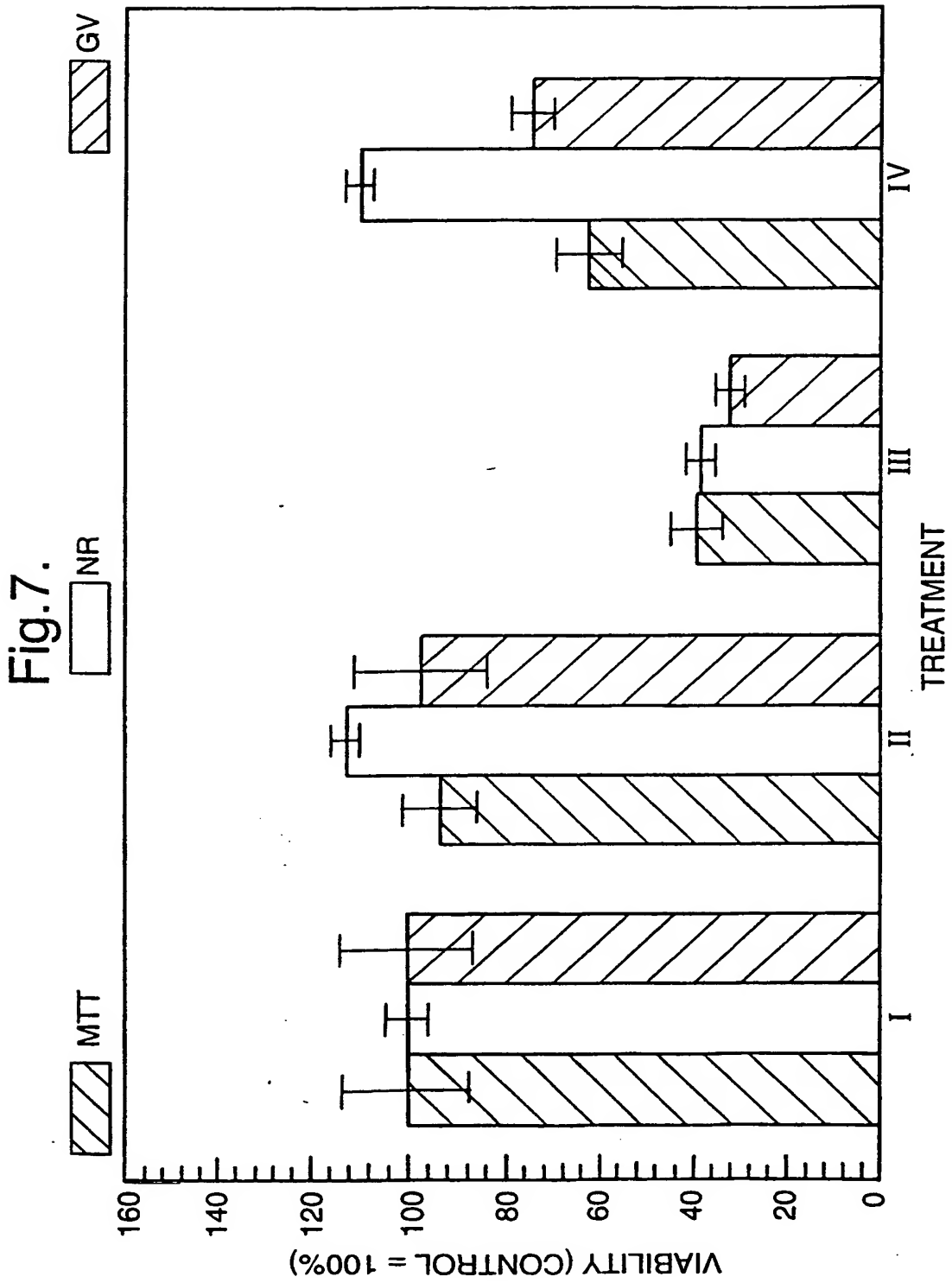


Fig.8.

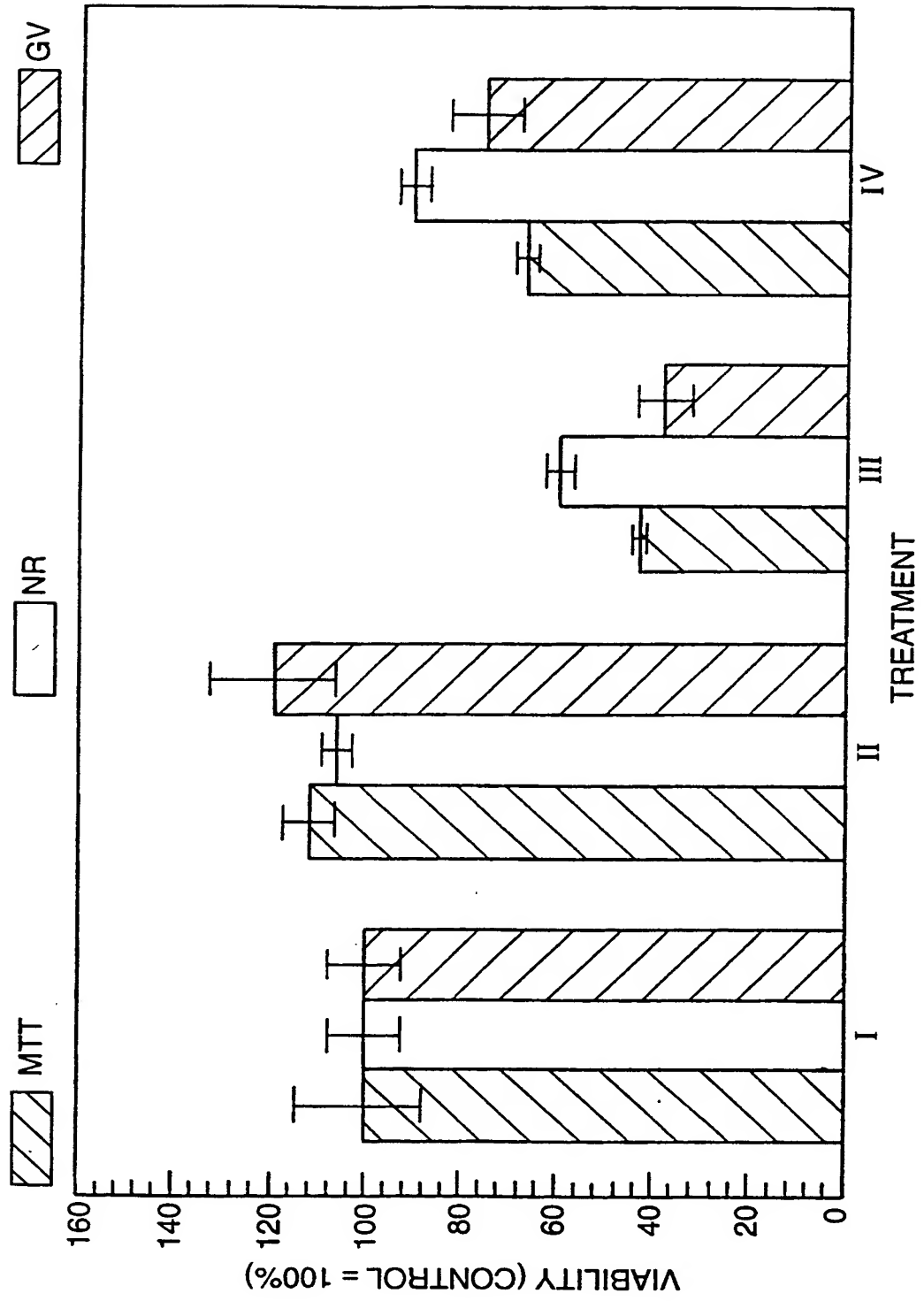
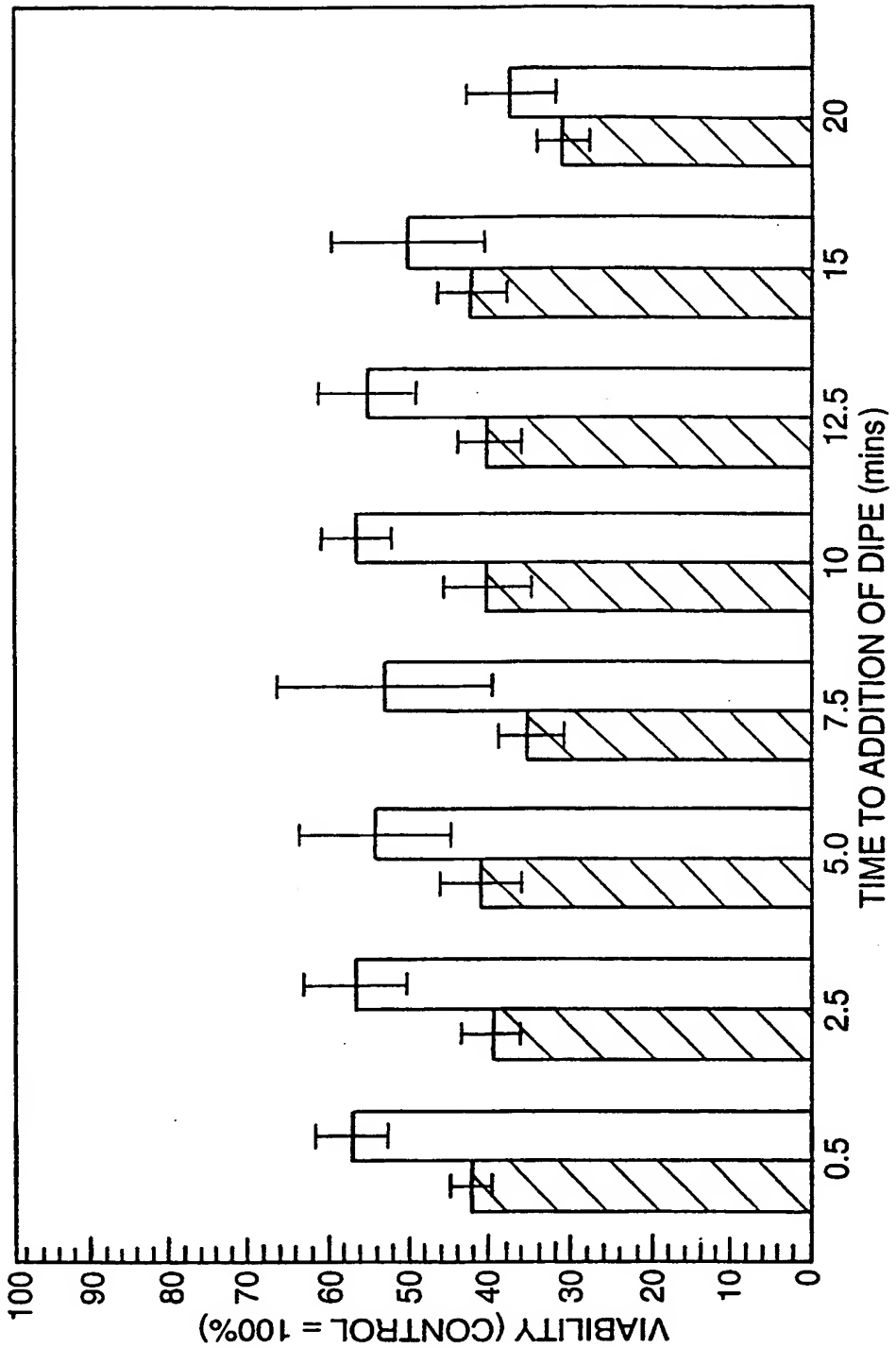


Fig.9.



INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 98/01603

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LINDSAY C D ET AL: "Monoisopropylglutathione ester protects A549 cells from the cytotoxic effects of sulfur mustard" HUM. EXP. TOXICOL. (HETOEA,09603271);1997; VOL.16 (11); PP.636-644, XP002079237 DERA, CBD Porton Down;Salisbury; SP4 0JQ; UK (GB)	1-4,6,8, 11-14
Y	see abstract see page 636, column 1, paragraph 1 - page 637, column 1, paragraph 2 see page 638, column 2, paragraph 2 - page 641, column 2, paragraph 1; figures 1,2,4	5,7,9, 10,15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1998	02/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel: (+31-70) 340-2040; Tx: 31 651 epo nl; Fax: (+31-70) 340-3016	A. Jakobs

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/01603

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 04024 A (UNITED KINGDOM SECRETARY OF STATE FOR DEFENCE, LONDON;UK) 19 March 1992 see page 5, line 39 - page 7, line 2; example 2; tables 1-4 ---	5,7,9, 10,15
X	AMIR A ET AL: "Glutathione protects J774 macrophage cells against sulfur mustard induced toxicity" MED. DEF. BIOSCI. REV., PROC. (64UTAN);1996; VOL.2,; PP.715-728, XP002079238 Israel Inst. Biological Research;Ness Ziona; Israel (IL) see abstract see page 716, paragraph 1-2; figures 1-6 see page 717, paragraph 3 - page 721, paragraph 4 ---	1-3,6, 10-14
X	BOYD M R ET AL: "Protective role of endogenous pulmonary glutathione and other sulfhydryl compounds against lung damage by alkylating agents. Investigations with 4-ipomeanol in the rat" BIOCHEM. PHARMACOL. (BCPCA6,00062952);1982; VOL.31 (8); PP.1579-83, XP002079240 Natl. Cancer Inst.;Div. Cancer Treat.; Bethesda; 20205; MD; USA (US) see abstract ---	1-3
X	TAYLOR Y C ET AL: "Elevation of intracellular glutathione levels following depletion and its relationship to protection against radiation and alkylating agents" PHARMACOL. THER. (PHTHDT,01637258);1988; VOL.39 (1-3); PP.293-9, XP002079242 Stanford Univ.;Med. Cent.; Stanford; 94305; CA; USA (US) see page 297, paragraph 2 - page 298, paragraph 1; figure 6 ---	1-3
X	ZUCALI J R ET AL: "Protective effects of human interleukin-1 on hematopoietic progenitor cells from L-phenylalanine mustard" ONCOL. REP. (OCRPEW);1995; VOL.2 (5); PP.851-6, XP002079239 Univ. Florida;Dep. Med.; Gainesville; 32610; FL; USA (US) see page 854, column 1, paragraph 2 - column 2, paragraph 1; figure 6 ---	1-3

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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/GB 98/01603

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	SEAGRAVE J C ET AL: "Zinc effects on glutathione metabolism relationship to zinc-induced protection from alkylating agents" BIOCHEM. PHARMACOL. (BCPCA6,00062952);1983; VOL.32 (20); PP.3017-21, XP002079241 Los Alamos Natl. Lab.;Genet. Groups; Los Alamos; 87545; NM; USA (US) see page 3020, column 2, paragraph 3	1-3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

....ernational application No.
PCT/GB 98/01603

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 11-13
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. tional Application No
PCT/GB 98/01603

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9204024 A	19-03-1992	AU 652183 B	18-08-1994
		AU 8525591 A	30-03-1992
		CA 2089158 A	01-03-1992
		EP 0546063 A	16-06-1993
		GB 2262446 A,B	23-06-1993
		JP 6500113 T	06-01-1994